

Replication and Recombination in Ligase-Deficient *rII* Bacteriophage T4D

H. M. KRISCH, D. B. SHAH, AND HILLARD BERGER

Departments of Biophysics and Biology, The Johns Hopkins University, Baltimore, Maryland 21218

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Deoxyribonucleic acid replication and genetic recombination were investigated after infection of *Escherichia coli* with ligase-deficient *rII* bacteriophage T4D. The major observations are: (i) deoxyribonucleic acid synthesis is discontinuous, (ii) the discontinuities are more slowly repaired than in wild-type infection, (iii) host ligase is required for viability, and (iv) genetic recombination is increased.

Karam (5) and Berger and Kozinski (1) have observed that amber mutations in the structural gene for polynucleotide ligase (gene 30) of phage T4D are no longer lethal when an *rII* mutation is present in the same phage. Physicochemical analysis of ³²P-labeled parental phage deoxyribonucleic acid (DNA) indicated that the *rII* mutation prevents the extensive endonucleolytic degradation of phage DNA which is normally observed with ligase-deficient *rII*⁺ phage (1). These results suggest that an endonuclease is responsible for the lethality associated with ligase-defective phage and that the *rII* mutation restores viability by directly or indirectly decreasing endonucleolytic action. This interpretation of the mechanism of suppression is consistent with the observation that ligase-deficient phage viability is restored when chloramphenicol is added 5 min postinfection and then removed 25 min later (1, 6, 8). Presumably, chloramphenicol during this phase of infection partially blocks expression of functions which allow endonucleolytic degradation of parental DNA. Limited DNA synthesis can nevertheless occur under these conditions, utilizing those enzymes synthesized before chloramphenicol addition (8).

In this communication, we report on the replication and recombination which occur after infection with a phage which is defective in both ligase and *rII* function.

MATERIALS AND METHODS

Phage stocks. The amber mutants of phage T4D, *amX39* (gene 30), *amN69* (gene 12), and *amB255* (gene 10), were kindly supplied by R. S. Edgar. The *rIIA* mutant, *r59*, was obtained from A. H. Doermann. The mutant *tsNK2* was isolated as a spontaneous pseudorevertant of *amX39* at 42°C in *Escherichia coli* B. The double mutant *amX39-tsNK2* does not grow at 30°C in *E. coli* B. Since T4D does not grow on bacterial strain TAU bar or TAU bar TS7, studies with

these strains utilized a T4D derivative designated T4Dγ. This derivative was isolated as a spontaneous mutant of T4D⁺ which had a high efficiency of plating and a nearly normal plaque morphology on these hosts. Phage stocks containing combinations of these markers were constructed by recombination.

Bacterial strains. *E. coli* B was used as the non-permissive host for in vivo and in vitro experiments with amber mutants. *E. coli* CR63 was used to prepare stocks and as the permissive host for phage containing amber mutations. The nonpermissive host for *rII* mutants was CR63(M). For studies on the role of host ligase in the restoration of viability to ligase-deficient phage, bacterial strains TAU bar and a ligase temperature-sensitive mutant TAU bar TS7 (12, 13), kindly provided by E. C. Pauling, were used.

Phage crosses. Crosses of *amX39-r59-amN69* by *amX39-r59-amB255* were carried out in *E. coli* B by the methods of Chase and Doermann (2). In each experiment, a minimum of 250 total progeny and 250 late function *am*⁺ recombinants were counted. Control crosses, *r59-amB255* by *r59-amN69*, were done simultaneously. For studies on the effect of chloramphenicol on recombination, crosses were carried out as above, but at the indicated times after infection the media were supplemented with 100 μg of chloramphenicol per ml (Parke, Davis & Co.). Chloramphenicol was removed to allow maturation by diluting 5,000-fold into unsupplemented media.

Cross lysates were plated on CR63 to determine total progeny and on *E. coli* W3110AB24 Su A (obtained from C. Yanofsky), which is restrictive for the late-function amber mutations used, to determine the frequency of wild-type late-function recombinants.

Physicochemical characterization of phage DNA. Methods were identical to those previously employed by Shah and Berger (J. Mol. Biol., *in press*).

RESULTS

Isolation and in vivo characterization of an *rII* temperature-sensitive mutant. To characterize the time course of the lethality of *rII* function in the absence of gene 30 product, a temperature-sensitive *rII* mutant was isolated. This mutant was

TABLE 1. *Phage production*

Phage mutant	Host bacterium	Burst size ^a at 30 C	Burst size ^a at 42.5 C
T4D ⁺	<i>E. coli</i> B	126	246
<i>amX39</i>	<i>E. coli</i> B	<0.01	<0.01
<i>amX39-tsNK2</i>	<i>E. coli</i> B	<0.01	179
<i>amX39 + amX39-tsNK2</i>	<i>E. coli</i> B	<0.01	<0.01
T4D ⁺	<i>E. coli</i> CR63	376	173
<i>amX39</i>	<i>E. coli</i> CR63	381	173
<i>amX39-tsNK2</i>	<i>E. coli</i> CR63	353	185
<i>amX39</i>	<i>E. coli</i> CR63(λh)	630	693 ^b
<i>amX39-tsNK2</i>	<i>E. coli</i> CR63(λh)	718	200 ^b

^a Burst sizes were calculated as the number of progeny per infected cell.

^b At 40 C.

selected as a spontaneous temperature-sensitive pseudorevertant of *amX39*. The doubly mutant strain has been designated *amX39-tsNK2*. It grows well on *E. coli* B at 42 C but does not grow at 30 C. Table 1 shows the burst size of the revertant and the parental *amX39* mutant in *E. coli* B, *E. coli* CR63, and *E. coli* CR63(λh) at 30 and 42 C. Since the double mutant grows equally well at high and low temperature on *E. coli* CR63, it is unlikely that the reversion event is due to a change of the amber codon in *amX39* to a codon which confers cold sensitivity to the ligase function. The growth response in CR63 also indicates that the temperature-sensitive mutation responsible for suppression does not reduce phage yields when there is normal ligase function. The ability of the revertant to grow nearly normally in *E. coli* CR63 (λh) at high temperature shows that this suppressor mutation does not have typical *rII* phenotype. Gene 30 suppressors of this type which are within the *rII* region have been described (1).

Confirmation of the *rII* site of *tsNK2* mutation was found by mapping it with respect to known *rII* mutations in both two- and three-factor crosses. The site of *tsNK2* was very close to the site of the marker *r59* (*rIIA*). Table 1 also shows that *tsNK2*, as with other previously described *rII* mutations, was recessive to the wild-type allele.

Since *tsNK2* suppresses gene 30 mutants only at high temperature, we have performed temperature-shift experiments to define the time at which *rII*⁺ function is lethal in the absence of phage ligase. Phage production after a temperature shift from 30 to 41 C at various times after infection of *E. coli* B with *amX39-tsNK2* indicates that incubation at 41 C must be initiated within 15 min after infection for the production of viable progeny (Fig. 1). This result is in good agreement with a similar experiment of J. D. Karam (*personal communication*).

DNA synthesis with *amX39-tsNK2*. The ob-

servation that gene 30 function is required for T4 viability has been interpreted to mean that phage-induced polynucleotide ligase is normally required for replication and cannot be replaced by bacterial ligase. It is clear, however, that the requirement for phage ligase is removed by the *rII* mutation, presumably by creating intracellular conditions which permit DNA synthesis by using only preexisting bacterial ligase. It has been previously shown that suppression of *amX39* by *rII* mutations prevents endonucleolytic degradation of parental DNA (1). This endonucleolytic attack of the input template molecule, before extensive replication has occurred, is probably the early lethal event which was demonstrated in the temperature-shift experiment (Fig. 1).

The DNA synthesized after infection with *amX39-tsNK2* has been examined on neutral and alkaline sucrose gradients. The results of these sedimentation studies of DNA labeled with ³H-thymidine for long times after infection with either *amX39-tsNK2* or *amX39* at 42 C are shown in Fig. 2. The DNA synthesized by the double mutant (*rII*-ligase-deficient) was mature-sized even when denatured; the gene 30 single mutant (*rII*⁺-ligase-deficient), however, synthesized only very short segments of DNA which failed to be covalently joined into mature molecules. Figure 3A shows results from an infection at 31 C with *amX39-tsNK2*; at this temperature, the double mutant behaved like the single mutant. The results obtained at 42 C were similar to those in Fig. 2C. Figure 3B shows the results of a mixed infection of *amX39-tsNK2* and *amX39* at 42 C; as expected, the *rII* mutation was recessive to the wild-type allele and only low-molecular-weight DNA was synthesized.

To characterize more completely the DNA synthesis under ligase-deficient conditions, the replicative intermediates were examined in pulse-labeling experiments. In accord with previous findings of Okazaki et al. (11) in wild-type infec-

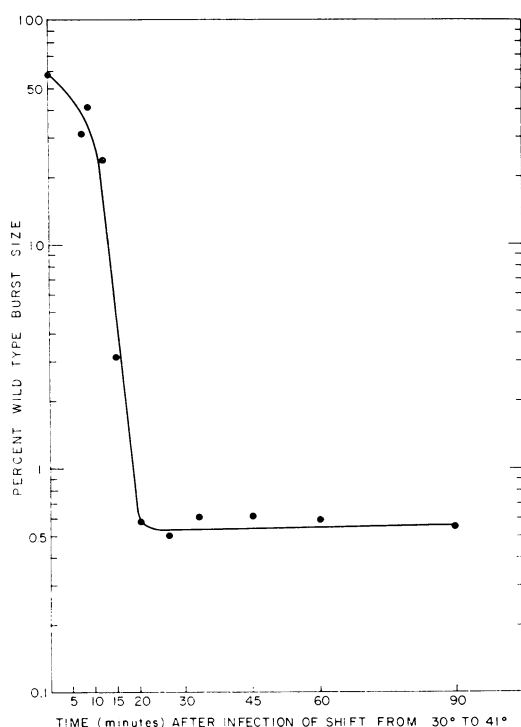


FIG. 1. Temperature-shift experiment. *E. coli* B cells were infected at 30 C with *amX39-tsNK2* at a multiplicity of 5 PFU per bacterium, and at the indicated times samples were diluted 20-fold into fresh medium at 41 C. Chloroform was added 90 min after infection to lyse the cells. Burst sizes are given as a percentage of those observed with *T4D*⁺-infected cells handled identically.

tion, DNA labeled with a short pulse sedimented in alkaline sucrose in a peak of approximately 8 to 10S; with longer pulse time, the bulk of the label was converted into mature-size molecules (Fig. 4A). In contrast (Fig. 4B), after infection with *amX39-tsNK2* at 42 C, short fragments were produced, but the rate of conversion of short fragments of newly synthesized DNA into mature-size molecules was markedly reduced. For example, after a 150-sec pulse more than 90% of the DNA from the wild type sedimented rapidly with marker DNA; under the same conditions in an *amX39-tsNK2* infection, the newly synthesized DNA was much more heterogeneous and generally of less than mature size. It should be noted that the amount of label incorporated during the pulse differed less than twofold between wild-type and *amX39-tsNK2*-infected cells.

Similar pulse-labeling experiments have been carried out with the double mutant *amX39-r59* used to infect *E. coli* B at 30 C. The *r59* marker

is a missense mutation within the *rIIA* cistron which has normal *rII* phenotype and is strongly restricted on λ lysogens. Results obtained in these experiments (not shown) were similar to those obtained with *amX39-tsNK2* infection at 42 C. Iwatsuki and Okazaki have obtained comparable results in unpublished experiments [cited in Iwatsuki and Okazaki, (4)] on the replicative intermediates after infection with ligase-deficient *rII* phage.

The above results indicate that after infection with ligase-deficient *rII* phage (i) DNA synthesis is discontinuous and (ii) the short fragments of DNA produced by discontinuous synthesis are slowly covalently linked to produce mature-size molecules. A likely explanation for these findings is that in the absence of *rII* function bacterial ligase joins the newly synthesized Okazaki fragments.

Role of bacterial ligase. To determine the role of bacterial ligase in the growth of ligase-deficient *rII* phage, we have used a bacterial strain, TAU bar TS7, which is temperature-sensitive and has low levels of temperature-sensitive polynucleotide ligase activity (12, 13). At 42 C, the mutant bacteria have less than 2 to 4% of wild-type levels of ligase (3). To get adequate phage growth on this bacterial strain or its parental strain, TAU bar, it was first necessary to isolate a mutant (*T4D* γ). The inability of wild-type *T4D* to grow in TAU bar is not understood; however, it is possible that TAU bar restricts *T4D*⁺ and that the phage mutant is less sensitive to restriction. *AmX39*, *amX39-r59*, and *amX39-tsNK2* containing the γ marker were constructed by recombination and tested for the ability to produce phage in the absence of host ligase. In the control experiments, TAU bar TS7 was infected with *T4D* γ and produced phage progeny. As would be expected, *amX39* γ , *amX39-tsNK2* γ , and *amX39-r59* γ were equally nonviable at 42 C in the absence of host ligase (Table 2). In contrast to this, the parental strain TAU bar, which produces normal bacterial ligase, did produce progeny after infection at 42 C with *amX39-tsNK2* γ or *amX39-r59* γ but not with *amX39* γ . Thus, it can be concluded that for ligase-deficient phage to replicate two criteria must be satisfied: (i) normal levels of host ligase must be present and (ii) the endonucleolytic attack of the DNA early in the infection must be prevented by inactivation of *rII* function.

Since these experiments have been carried out, a new ligase-deficient bacterial strain has been isolated (3). This strain, which has reduced levels of ligase activity in enzyme assays, differs from the strain used here in several aspects. In

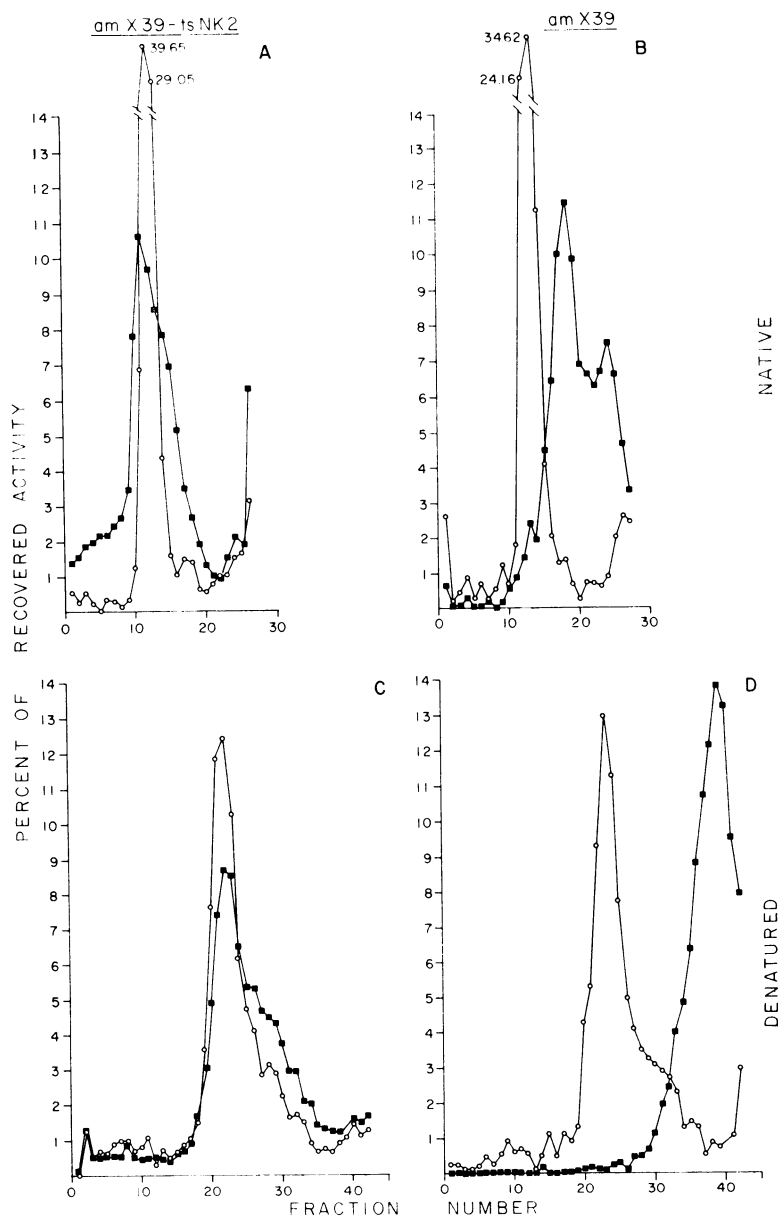


FIG. 2. Integrity of newly synthesized intracellular DNA: neutral and alkaline sucrose gradients. *E. coli* B cells were infected at 42.5 C with amX39-tsNK2 (A and C) or amX39 (B and D) at a multiplicity of 7.5 PFU per bacterium. After 6 min, TCG medium (14) was supplemented with 2 μ g of thymidine per ml, 5 μ g of fluorodeoxyuridine per ml, and 20 μ g of uracil per ml; one minute later ^3H -thymidine (12.5 $\mu\text{Ci/ml}$) was added. At 15 min postinfection, samples of infected cells were lysed in the presence of ^{32}P -labeled phage by the lysozyme-Sarkosyl-high temperature method. Samples were centrifuged for 150 min at 86,000 \times g at about 10 C through 5 to 20% neutral (A and B) or alkaline (C and D) sucrose gradients. Sedimentation is from right to left in this and subsequent figures. Symbols: ■, ^3H -labeled DNA; ○, ^{32}P -labeled T4 marker DNA.

vivo it shows no deficiency in joining of newly synthesized Okazaki fragments to form chromosomal DNA. Unlike TAU bar TS7, this strain does not show aberrant growth characteristics

under nonpermissive conditions. In spite of these profound differences in phenotype from the TS7 mutant, this strain, when used as a host for ligase-deficient π II phage, is also unable to produce

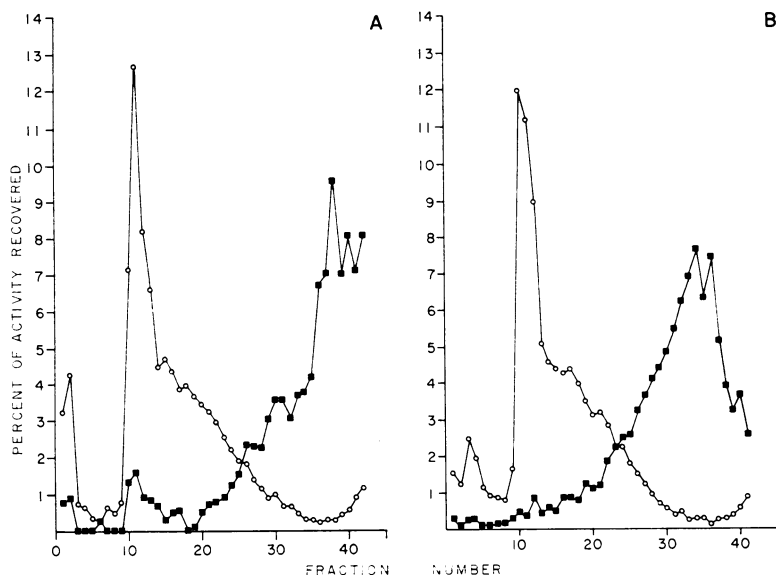


FIG. 3. Integrity of newly synthesized DNA: alkaline sucrose gradients. *E. coli* B cells were infected in TCG medium supplemented with 200 μ g of 2'-deoxyadenosine per ml with either amX39-tsNK2 (multiplicity of infection 15) at 31 C (A) or amX39-tsNK2 and amX39 (multiplicity of infection 7.5 each) at 42 C (B). At 7.5 min after infection, 12.5 μ Ci of 3 H-thymidine (7 Ci/mmole) per ml was added. Infected cells were labeled for 8 min and then lysed in the presence of 32 P-labeled phage by the lysozyme-Sarkosyl-low temperature method. Samples were centrifuged for 100 min at $189,000 \times g$ at 5 C through 5 to 20% alkaline sucrose gradients. Symbols: \blacksquare , 3 H-labeled DNA; \circ , 32 P-labeled T4 marker DNA.

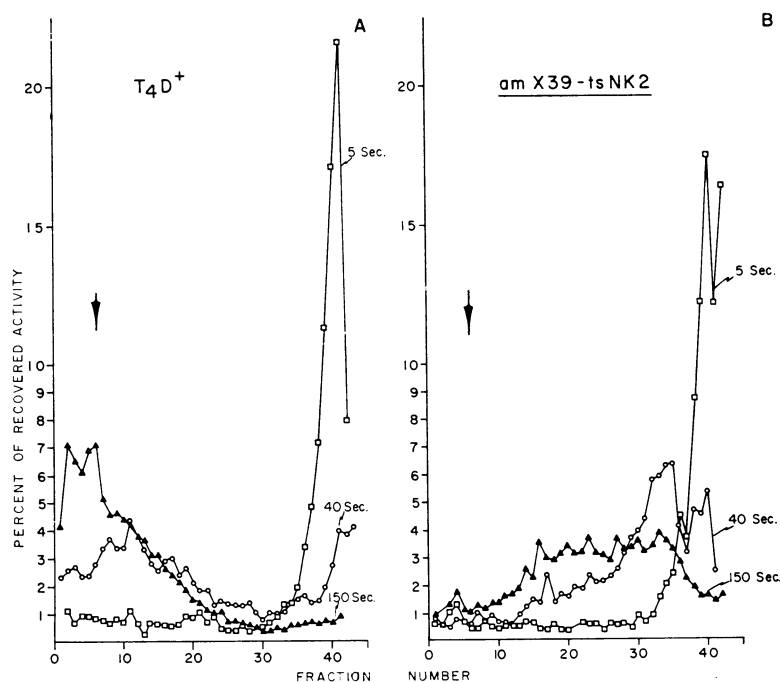


FIG. 4. Short-pulse-labeling experiment: alkaline sucrose. *E. coli* B was infected with T4D⁺ (A) or amX39-tsNK2 (B) in TCG medium supplemented with 200 μ g of 2'-deoxyadenosine per ml at a multiplicity of 7.5 PFU per bacterium. After 15 min, 3 H-thymidine (7 Ci/mmole) was added (50 μ Ci/ml for a 5-sec pulse; for longer labeling times, 12.5 μ Ci/ml). The pulse was terminated by lysing infected cells in the presence of 32 P-labeled phage by the lysozyme-Sarkosyl-low temperature method. Samples were centrifuged for 129 min at $189,000 \times g$ at 5 C through 5 to 20% alkaline sucrose gradients. Sedimentation is from right to left. The arrow indicates the position of 32 P-labeled T4 marker DNA. Symbols: \square , 5-sec pulse, 3 H-labeled DNA; \circ , 40-sec pulse, 3 H-labeled DNA; \blacktriangle , 150-sec pulse, 3 H-labeled DNA.

TABLE 2. Growth in *E. coli* TAU bar and TAU bar TS7 of T4D mutants deficient in ligase

Phage mutant	Host bacterium	Burst size ^a at 30 C	Burst size ^a at 41 C
T4D γ	TAU bar	23.2	18.2
<i>amX39</i> - γ	TAU bar	0.06	0.02
<i>amX39-r59</i> - γ	TAU bar	3.0	6.0
<i>amX39-tsNK2</i> - γ	TAU bar	0.1	6.9
T4D γ	TAU bar TS7	109	16.5
<i>amX39</i> - γ	TAU bar TS7	0.01	<0.01
<i>amX39-r59</i> - γ	TAU bar TS7	5.0	<0.02
<i>amX39-tsNK2</i> - γ	TAU bar TS7	0.25	0.01

^a Burst sizes were calculated as the number of progeny per infected cell.

viable progeny. Thus, the ability to support growth of ligase-deficient *rII* phage appears to be a sensitive in vivo assay for host ligase levels.

Effect of limited ligase on phage genetic recombination. The preceding analyses of the replicating DNA after infection with ligase-deficient *rII* phage indicate that the newly replicated DNA exists in a highly nicked form for appreciably longer times than after infection with wild-type phage. As shown in Table 3, genetic recombination between a pair of linked genes was increased almost sevenfold under these conditions, suggesting a possible relationship between the persistence of nicks in the intracellular DNA and the degree of genetic recombination in the progeny.

An alternative explanation of the observed increases in recombination is suggested by two other manifestations of the ligase-deficient *rII* phenotype. As can be seen in Fig. 5, DNA synthesis lagged by approximately 10 min compared to infection with ligase-positive *rII* phage. Concomitant with the lag in DNA synthesis was a similar, or slightly more pronounced, delay in the appearance of mature phage (Fig. 6). Since the DNA molecules are spending a longer time in the vegetative state, this could be expected to result in some increases in recombination frequency (9). To test this possibility, delays in maturation were artificially introduced by the addition of chloramphenicol and the effect on recombination was measured. It is clear from the results of such an experiment (Table 4) that temporary chloramphenicol-induced blockage of late function expression fails to produce significant alterations in the frequency of recombinants.

In these experiments, chloramphenicol was added at a variety of times after infection. Kozinski, Kozinski, and Shannon (7) have shown that chloramphenicol addition after 9 min at 37 C has no effect on the transfer of parental label to progeny DNA molecules. Mattson (10) has also reported that chloramphenicol-induced delays in

TABLE 3. Effect of limited ligase on genetic recombination

Cross	Per cent recombination ^a between <i>amB255</i> and <i>amN69</i>
1. <i>r59-amB255</i> \times <i>r59-amN69</i> (control).....	3.4, 3.9
2. <i>amX39-r59-amB255</i> \times <i>amX39-r59-amN69</i> (ligase-deficient).....	26.4, 23.0

^a See Materials and Methods. The percentages were calculated by multiplying by two the frequency of wild-type recombinants. Values are given for two separate crosses.

maturation have no effect on the accumulation of recombinants.

DISCUSSION

Our results indicate that replication producing mature-size phage DNA molecules can occur in the absence of the gene 30 product utilizing only the host ligase. Limitation of an endonucleolytic activity controlled by the *rII* genes of T4D is necessary for such replication to take place. Apparently the early endonucleolytic attack of injected DNA molecules cannot be repaired adequately by host ligase, thus accounting for the lethality of ligase-deficient *rII*⁺ phage.

It has been previously reported that chloramphenicol addition at early times after infection with *amX39* results in partial suppression of the ligase-deficient phenotype and DNA of high-molecular-weight is synthesized. In the experiments reported here and in previous experiments with ligase-deficient *rII* phage (1), the *rII* mutation has an effect very similar to chloramphenicol in suppressing the abnormal DNA synthesis phenotype of the gene 30 mutant. Preliminary experiments suggest that the mechanism of chloramphenicol rescue of gene 30 mutants is by

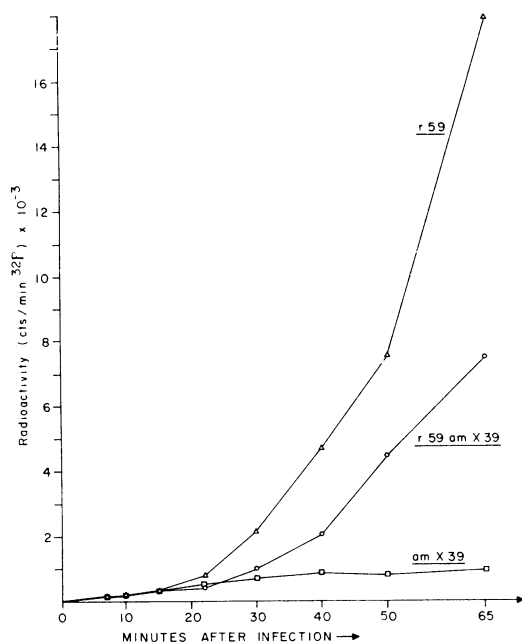


FIG. 5. Net DNA synthesis in phage-infected *E. coli* B at 30°C. ^{32}P incorporation into acid-insoluble, alkali-resistant material. Infection was carried out in TCG medium with a multiplicity of 8 phage per bacterium. ^{32}P (1 $\mu\text{Ci}/\text{ml}$) was added 3 min after infection. Symbols: Δ , *r59*; \circ , *r59-amX39*; \square , *amX39*.

TABLE 4. Effect of chloramphenicol-induced delays in maturation on genetic recombination

Cross	Chloramphenicol treatment	Per cent recombination ^a between <i>amB255</i> and <i>amN69</i>
1. <i>r59-amB255</i> \times <i>r59-amN69</i>	None	3.9
Same	7-37 min	3.7
Same	10-40 min	3.2
Same	13-43 min	3.3
Same	17-47 min	2.9
2. <i>amX39-r59-amB255</i> \times <i>amX39-r59-amN69</i> (ligase-deficient)	None	23.0

^a See Materials and Methods. The percentages were calculated by multiplying by two the frequency of wild-type recombinants.

its inhibition of the synthesis of the *rII* gene product (H. Krisch, unpublished data).

Once ligase-deficient *rII* replication has begun with the relatively un-nicked parental DNA as a template (1), it proceeds in a discontinuous

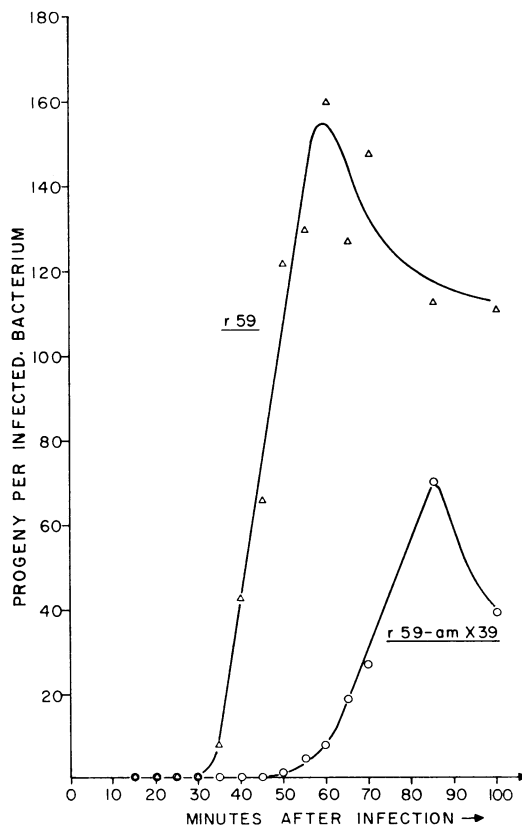


FIG. 6. One-step growth curve with premature lysis. *E. coli* B was infected with either *r59* or *amX39-r59* at 30°C at a multiplicity of 7.5 PFU per bacterium. At the indicated time, samples of the phage-infected cells were removed and lysed with chloroform and then titered on *E. coli* CR63. Data are given as progeny produced per infected bacterium. Symbols: Δ , *r59*; \circ , *amX39-r59*.

fashion. The replicative discontinuities which are produced are only slowly repaired by rate-limiting levels of host ligase. The pattern of DNA synthesis in ligase-deficient *rII* infection is thus compatible with the Okazaki model of DNA synthesis. The major difference from wild-type infection is the increased lifetime of the discontinuities in the newly replicated DNA molecules.

The increases in genetic recombination observed under these conditions are probably explained by the persistence of replicative DNA intermediates which contain numerous single-stranded nicks. Such structures may facilitate break-reunion recombination. The slight delay in both DNA synthesis and the production of mature viruses after ligase-deficient *rII* infection may also have effects on the formation of recombi-

nants. The results of the chloramphenicol experiment indicate that maturation delays of the observed duration are in themselves inadequate to account for effects of this magnitude. However, it cannot be ruled out that the prolonged incubation of the nicked vegetative DNA molecules has some contributory role in producing increased levels of recombination. More detailed studies of this altered recombination in ligase-deficient *rII* infection will be published elsewhere. Preliminary experiments suggest that the increases in recombination are less pronounced when the markers used in the cross are separated by very short intervals. This result is what would be expected if the markers are so close to each other that the probability of a replicative discontinuity occurring between them is small.

It is unclear from the results reported here whether replicative discontinuities are involved in the formation of recombinants after infection with wild-type T4D. It is possible that only under conditions of limited ligase do they have a significant role in this process. Nevertheless, our results probably justify a reconsideration of the importance of replicative discontinuities in current models of recombination.

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